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BIOTIC AND ABIOTIC STRESS

Overexpressed *glutamine synthetase* gene modifies nitrogen metabolism and abiotic stress responses in rice

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Abstract Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme in nitrogen metabolism; it catalyzes the critical incorporation of inorganic ammonium into glutamine. Two full-length cDNAs that encode the rice (*Oryza sativa*) cytosolic *glutamine synthetase1* genes (*OsGS1;1* and *OsGS1;2*) were isolated from a Minghui 63 normalized cDNA library, and *glnA* encoding GS in *Escherichia coli* was isolated by PCR amplification. Transformants for *GS* gene (*GS1;1*, *GS1;2*, and *glnA*) in rice were produced by an *Agrobacterium tumefaciens*-mediated transformation method, and transcripts of *GS* gene accumulated at higher levels in the primary transgenic plants. Our results indicated an increased metabolic level in GS-overexpressed plants, which showed higher total GS activities and soluble protein concentrations in leaves and higher total amino acids and total nitrogen content in the whole plant. Decreases in both grain yield production and total amino acids were observed in seeds of GS-overexpressed plants compared with wild-type plants. In addition, *GS1;2*-overexpressed plants exhibited resistance to Basta selection and higher sensitivity to salt, drought, and cold stress conditions, whereas the other two types of GS-overexpressed plants failed to show any significant changes for these stress conditions compared with wild-type plants.

Keywords *Glutamine synthetase* gene · Nitrogen metabolic level · Yield · Abiotic stress · Rice

Abbreviations

GS	Glutamine synthetase
GOGAT	Glutamate synthase
AS	Asparagine synthetase
Gln	Glutamine
Glu	Glutamate
Asn	Asparagine
Asp	Aspartate
Ala	Alanine
2-OG	α-Ketoglutarate
rbcS	Rubisco small subunit
γ-GHA	γ-Glutamylhydroxamate
PPT	Phosphinothricin
MS	Murashige and Skoog

Introduction

Nitrogen is a crucial macronutrient that is essential and rate-limiting for the growth and development of plants. Because of its strong impact on plant productivity, a large amount of nitrogenous fertilizer is applied to fields to maximize crop yields. However, nitrogenous fertilizer severely pollutes the environment, especially aquatic ecosystems. Therefore, increasing the crop nitrogen use efficiency through breeding programs, particularly in cereals, would allow less nitrogenous fertilizer to be applied while producing higher yield with better protein content. Achieving this aim requires a better understanding of nitrogen metabolism and its regulation and the

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identification of target genes for manipulation by either direct gene transformation or marker-assisted breeding (Miflin and Habash 2002). In higher plants, inorganic nitrogen from the soil is initially converted into organic nitrogen by two enzymes, glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.14 and EC 1.4.7.1). GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate (Glu) to yield glutamine (Gln); GOGAT transfers the amide group of Gln to α-ketoglutarate (2-OG) to subsequently produce Glu (Temple et al. 1998; Ireland and Lea 1999). Gln then serves as one of nitrogen donors for the biosynthesis of organic nitrogenous compounds, such as amino acids, nucleotides, and chlorophyll. Thus, the GS enzyme is likely to be a key factor controlling plant nitrogen assimilation.

One important characteristic of GS is its high affinity for NH_4^+ and thus its ability to incorporate NH_4^+ efficiently into organic compounds. GS enzymes are often found as multiple isoenzyme forms located both in the cytosol (GS1) and chloroplast/plastid (GS2) that play distinct roles (Ireland and Lea 1999; Lancien et al. 2000). Cytosolic GS1 plays an important role in primary nitrogen assimilation in root, and functions to generate Gln for transport in the phloem in the stem; chloroplastic GS2 plays a crucial role in reassimilation of NH_4^+ released via photorespiration in plants (Wallsgrove et al. 1987). In higher plants examined to date, there is a single nuclear gene for chloroplastic GS2; however, in soybean (*Glycine max*) and alfalfa several *GS2* genes have been identified (Zozaya-Garza and Sengupta-Gopalan 1999). In contrast, multiple homologous but distinct genes were found for cytosolic *GS1* (Tingey and Corruzi 1987; Li et al. 1993; Ireland and Lea 1999; Oliveira and Coruzzi 1999; Yamaya and Oaks 2004; Canovas et al., 2007; Hirel et al. 2007), and there are three members were identified in rice (*Oryza sativa*; *OsGS1;1*, *OsGS1;2*, and *OsGS1;3*; Ishiyama et al. 2004; Tabuchi et al. 2005, 2007). Among the three *GS1* genes in rice, *GS1;1* was expressed in all organs (i.e., root, leaf blade, leaf sheath, and spikelet), with higher expression in the leaf blade during the vegetative stage (Tabuchi et al. 2005). *GS1;2* transcripts were also detected in all organs, with higher expression in the root at the seedling stage, while *GS1;3* was specifically expressed in the spikelet (Tabuchi et al. 2007).

Based on the GS expression patterns and functions, a straightforward strategy for engineering nitrogen assimilation would be to enhance the GS enzyme activities in plants. Previous attempts at overexpressing the *GS* genes to improve plant nitrogen assimilation presented mixed results. Accelerated growth rate was observed in transgenic *Lotus corniculatus* plants overexpressing a soybean (*G. max*) GS1 isoenzyme driven by a *CaMV* 35S promoter (Vincent et al. 1997). Vegetative growth and photosynthetic capacity improvements were reported for cytosolic GS1-overexpressed poplar trees (Gallardo et al. 1999; Migge et al. 2000; Pascual et al. 2008) and tobacco (Fuentes et al. 2001; Oliveira et al. 2002). In addition, earlier flower and seed development were observed in transgenic wheat lines containing *Phaseolus vulgaris GS1* under the control of the Rubisco small subunit (*rbc*S) promoter (Habash et al. 2001).

Numerous studies of the GS enzyme have justified the importance of this enzyme in plant nitrogen metabolism. Particular attention has been devoted to studies on GS transformation in higher plants, which is expected to be a good molecular method to analyze gene functions and obtain transgenic plants with higher nitrogen use efficiency. The purpose of this study is to determine whether overexpressing *GS* can improve the nitrogen use efficiency or abiotic stress tolerance in transgenic rice plants. In this study, transgenic plants with the *CaMV* 35S promoter driving a rice *GS1;1*, a rice *GS1;2*, and an *Escherichia coli glnA* displayed an enhanced metabolic level quantified by increases in leaf total GS activities and soluble protein concentrations and higher total amino acids and total nitrogen contents in whole plants. Decreases in both grain yield production and total amino acids in seeds were identified in GS-overexpressed plants compared with wild-type plants. In addition, *GS1;2*-overexpressed plants exhibited resistance to Basta selection, but higher sensitivity to salt, drought, and cold stress conditions. Our findings provide a better understanding of nitrogen assimilation and abiotic stress, as overexpressing *GS* genes led to changes in rice nitrogen metabolism and also growth phenotype under abiotic stress conditions, and suggest that increasing the nitrogen use efficiency can be achieved by manipulating the specific GS isoenzymes in transgenic crop plants.

Materials and methods

Constructs and transformation

"Glutamine synthetase, rice" was used as a keyword to search the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and two mRNA sequences encoding rice cytosolic *GS1;1* (AB037595) and *GS1;2* (AB180688) were found. Two homologous rice full-length cDNA clones (EI#03-A15 to X14244 and EI#113-I02 to X14245) were found for these two sequences in the Minghui 63 normalized cDNA library of our laboratory (http://www.redb.ncpgr.cn; Chu et al. 2003). Two cDNA sequences isolated from the normalized cDNA library were digested by *Bam*HI and *Kpn*I enzymes, and *glnA* encoding *E. coli* GS was amplified by PCR using

the forward primer 5′-TGCTGGTACCATGTCCGCTGAA CACGTACT-3′ and reverse primer 5′-GATCTCTAGA GTGAATGTGCTTGCCACCGA-3′ which contained the enzyme site of *Kpn*I and *Xba*I, respectively. Three *GS* fragments (named *GS1;1*, *GS1;2*, and *glnA*) were then ligated into the *pCAMBIA 1301S* vector, driven by cauliflower mosaic virus (*CaMV*) 35S promoter and nopaline synthase 3′ terminator (Nos polyA). Standard molecular techniques (Sambrook et al. 1989; Maniatis et al. 1992) were used for DNA manipulation. The chimeric gene was transformed into the *japonica* rice cultivar Zhonghua 11 by an *Agrobacterium tumefaciens*-mediated transformation method (Hiei et al. 1994).

Northern blot and Southern blot analyses

Total RNA was extracted from the leaves with TriZol reagent (Invitrogen, Germany) according to the manufacturer's instructions; 15 μg of total RNA was then used for Northern blot analysis. Genomic DNA was extracted from T_0 transgenic plants by the CTAB method, and 4 μg of genomic DNA was digested by *Hin*dIII. The DNA was then transferred to a Hybond N+ nylon membrane (Amersham, Buchinghamshire, UK) for Southern blot analysis. Hybridizations were performed as described by Sambrook et al. (1989), with ^{32}P-labeled partial specific *GS* gene fragment of rice *GS1* and *E. coli glnA*, and ^{32}P-labeled partial DNA fragment of the *hygromycin* genes as a probe for Northern and Southern blot analysis, respectively. The results were detected by autoradiography.

Plant growth conditions

For determination of the phenotypes, the total GS activities and soluble protein concentrations in leaves, and the total amino acids and total nitrogen contents in whole plants of GS transformants, both GS-overexpressed plants and wild-type Zhonghua 11 plant seeds were germinated, sowed in sand, and transferred to a culture solution at the two-leaf stage. Both the normal nutrient solution [1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM Na_2SiO_3, 50 μM Fe–EDTA, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μM H_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$, 40.5 μM citric acid (pH 5.5) (Yoshida et al. 1976)] and the low-nitrogen nutrient solution (same composition as the normal nutrient solution except for 0.1 mM NH_4NO_3) were used in this study. The culture solution was refreshed once every 3 days. Along with this, GS-overexpressed plants and wild-type Zhonghua 11 plants were also grown under low-nitrogen field conditions for the analysis of free NO_3^-, free NH_4^+, and free amino acids in leaves; grain yield production; and total amino acids in seeds.

Metabolite analyses

The metabolite analyses were performed on the flag leaves of 28-day-old plants (a mixture of six flag leaves selected randomly).

For soluble protein analysis, plant materials were homogenized by grinding the freshly harvested leaves on ice with extraction buffer [10 mM Trizma (pH 7.5), 5 mM sodium glutamate, 10 mM $MgSO_4$, 1 mM dithiothreitol, 10% (v/v) glycerol, and 0.05% (v/v) Triton X-100]. The homogenates were then centrifuged at 12,000×*g* for 20 min at 4°C (Melo et al. 2003). The soluble protein concentration of the extract was measured by the Bradford (1976) protein assay using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA); bovine serum albumin was used as the standard protein.

For assessment of total GS enzyme activities, freshly harvested leaves were ground on ice with extraction buffer consisting of 70 mM MOPS (pH 6.8); 10 mM $MgSO_4$; 2 mM dithiothreitol; 5 mM glutamate; 0.1% (v/v) Triton X-100, and 10% (v/v) ethanediol. Semi-synthetase activity of GS was assayed, with NH_2OH used as an artificial substrate, by quantifying the formation of γ-glutamylhydroxamate (γ-GHA; O'Neal and Joy 1973). The homogenates were centrifuged at 12,000×*g* for 30 min at 4°C, and the supernatant was analyzed for total GS activities. Total leaf GS activities were measured in a preincubated assay buffer (37°C) consisting of 70 mM MOPS (pH 6.8), 100 mM glutamate, 50 mM $MgSO_4$, 15 mM NH_2OH, and 15 mM ATP. The reaction was terminated after 30 min at 37°C by adding acidic $FeCl_3$ solution (88 mM $FeCl_3$, 670 mM HCl, and 200 mM trichloroacetic acid). After allowing 10 min for the color to develop, the reaction mixture was centrifuged at 4,000×*g* at room temperature for 10 min, and 200 μl of supernatant was then transferred from each well into a new tube. The absorbency was measured in a spectrophotometrically quantification reader at 540 nm (Husted et al. 2002).

For free NO_3^- and NH_4^+ analysis, plant materials were homogenized by grinding the fresh harvested leaves in cold extraction buffer [50 mM Tris–HCl (pH 7.0), 10 mM imidazole, and 0.5% (w/v) β-mercaptoethanol]; the homogenates were then centrifuged at 12,000×*g* for 20 min at 4°C (Oliveira et al. 2002). Free NO_3^- in the supernatant was determined by using the Griess method (Walther et al. 1999), the absorbance at 540 nm was determined and NO_3^- contents were calculated from the standard curve of KNO_3. Free NH_4^+ in the supernatant was determined by the Berthelot color reaction method (Gordon et al. 1978), the absorbance at 480 nm was determined, and NH_4^+ contents were calculated from the standard curve of NH_4NO_3.

Free amino acids and total amino acids were analyzed by a Hitachi amino acid analyzer (L-8800, Hitachi

Instruments Engineering, Japan) according to the manufacturer's instructions, and leucine was used as a standard. Total nitrogen content was analyzed using flow injection analyzer (FIA-3700, Flow Injection Analysis, Germany) according to the manufacturer's instructions.

Results

Accumulated GS mRNA transcripts in T_0 transgenic plants

Two homologous but distinct mRNA sequences of rice cytosolic isoenzymes of GS (named GS1;1 and GS1;2, 83% nucleotide homology and 85% amino acid homology within the coding region) were found in NCBI GenBank database, and two EST clones (EI#03-A15 and EI#113-I02) encoded GS1;1 and GS1;2 polypeptides were obtained by screening the Minghui 63 normalized cDNA library of rice. Two full-length cDNAs of the rice *GS1* genes digested from EI#03-A15 and EI#113-I02 EST clones and *glnA* amplified from the *E. coli* genome were individually ligated to the vector *pCAMBIA 1301S*, which contained a *hygromycin* resistance gene and were driven by the *CaMV* 35S promoter. The ligated gene fragments were then transformed into Zhonghua 11 (rice japonica cultivar) by the *Agrobacterium*-mediated transformation method (Fig. 1a). For each constructs, more than 30 independent T_0 transgenic plants were generated, and more than 85% were positive transformants as detected by PCR of *hygromycin* resistance gene. Southern blot analysis suggested that all of the independent positive transgenic plants had one to six copies of T-DNA (portion of the Ti plasmid that is transferred to plant cells; data not shown). Northern blot analysis of the transgene in 6–12 independent positive transgenic plants with single copy number showed that five *GS1;1* transgenic plants (31, 40, 42, 43, 44), five *GS1;2* transgenic plants (5, 6, 7, 8, 10), and six *glnA* transgenic plants (20, 22, 56, 59, 110, 118) had higher levels of transgene expression, whereas the others did not overexpress the transgene (Fig. 1b).

To examine the phenotypes of transgenic lines, T_1 progeny of the three types of GS-overexpressed plants and wild-type plants were grown hydroponically in normal or low-nitrogen nutrient solution. The plants were harvested at the vegetative stage (up to 6 weeks after germination) to measure leaf total GS activities, fresh weight, and dry weight. Significant (*t*-test, $P < 0.05$) increases in the leaf total GS activities of the GS-overexpressed plants under both normal nitrogen and low-nitrogen conditions were observed (18% for *GS1;1*-, 19% for *GS1;2*- and 25% for *glnA*-overexpressed plants under normal nitrogen condition; 36% for *GS1;1*-, 44% for *GS1;2*-, and 46% for *glnA*-overexpressed plants under low-nitrogen condition;

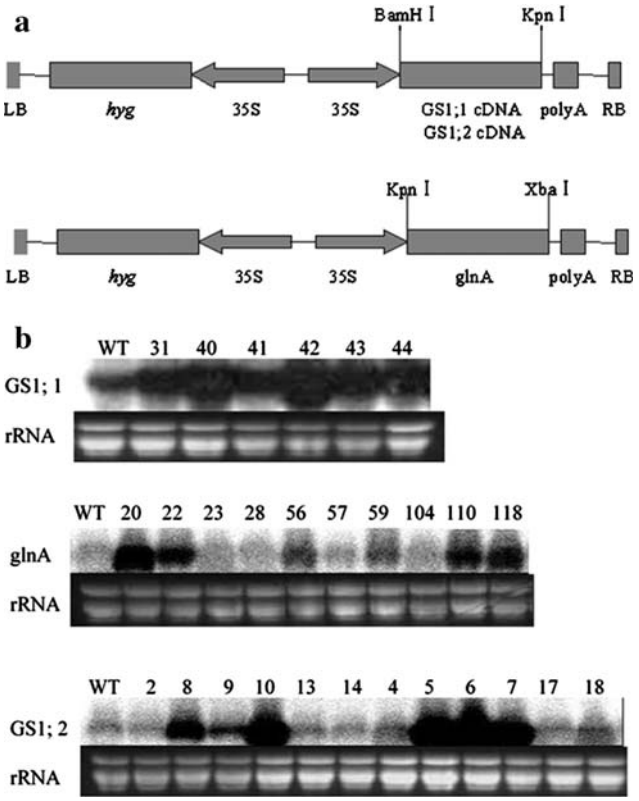

Fig. 1 Generation of transgenic rice plants that overexpress *GS* genes (*GS1;1*, *GS1;2*, and *glnA*). **a** The constructs of the plasmid containing a derivative of the *CaMV* 35S promoter (35S), *GS*, and the poly A terminator between the right (*RB*) and the left (*LB*) borders of the T-DNA. The *hygromycin* resistance gene (*hyg*) was located between the LB and the 35S promoter. **b** Northern blot analysis of GS mRNA transcriptional levels in GS transgenic plants of the T_0 generation and the wild-type plants (*WT*). The results reported in the figures are three *GS1;1*-overexpressed plant lines (31 C1, 42 C3, and 43 C4), three *GS1;2*-overexpressed plant lines (5 A1, 7 A3, and 8 A4), and three *glnA*-overexpressed plant lines (59 B2, 110 B3, and 118 B4)

Fig. 2a), although the increase in enzyme activities was smaller than the increase in the corresponding transcripts. Analysis of fresh weight and dry weight failed to show significant differences between GS-overexpressed and wild-type plants under both normal and low-nitrogen conditions (data not shown here).

Higher nitrogen metabolic status in GS-overexpressed plants

To evaluate the effects of higher GS mRNA transcriptional levels on nitrogen assimilation, we analyzed the leaf soluble protein concentrations of GS-overexpressed plants in the T_2 generation grown hydroponically in normal or low-nitrogen nutrient solution and the free NO_3^-, free NH_4^+, and free amino acid concentrations of GS-overexpressed plant leaves in the T_2 generation grown under low-nitrogen field conditions. There was a significant ($P < 0.05$) increase

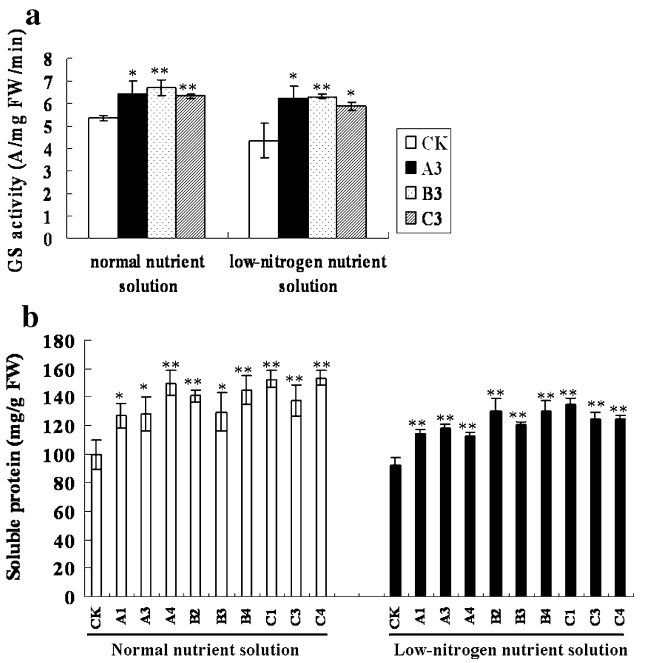

Fig. 2 Higher total GS activities and soluble protein concentrations in GS-overexpressed plants. **a** Total GS activities in flag leaves of the wild-type plant (*CK*) and transgenic plants overexpressing *GS1;2* (*A3*), *glnA* (*B3*), and *GS1;1* (*C3*), grown hydroponically in normal nutrient solution or low-nitrogen nutrient solution for 4 weeks before harvest. **b** Soluble protein concentrations in flag leaves of CK and transgenic plants overexpressing *GS1;2* (*A1*, *A3*, *A4*), *glnA* (*B2*, *B3*, *B4*), and *GS1;1* (*C1*, *C3*, *C4*), grown hydroponically in normal nutrient solution (*white bars*) and low-nitrogen nutrient solution (*black bars*) for 4 weeks before harvest. Values are mean ± SD from three independent experiments using six randomly mixed plant flag leaves. *, **Significant differences at the levels of $P = 0.05$ and $P = 0.01$, respectively

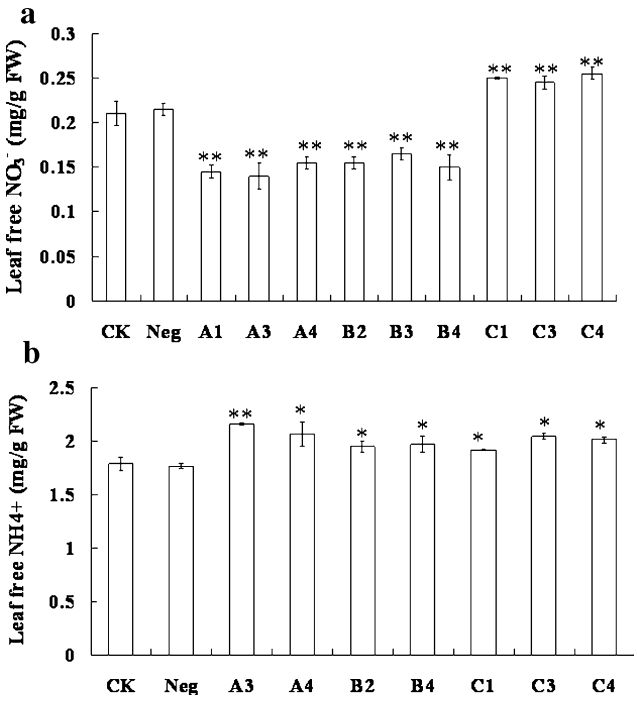

Fig. 3 Metabolite contents in the control plants and GS-overexpressed plants. **a** Free NO_3^- concentration and **b** free NH_4^+ concentration in the flag leaves of wild-type (*CK*), negative control (*Neg*, transgenic plants with no GS overexpressing), and transgenic plants overexpressing *GS1;2* (*A1*, *A3*, *A4*), *glnA* (*B2*, *B3*, *B4*), and *GS1;1* (*C1*, *C3*, *C4*), grown under low-nitrogen field conditions for 4 weeks before harvest. Values are mean ± SD from three independent experiments using six randomly mixed plant flag leaves. *, **Significant differences at the levels of $P = 0.05$ and $P = 0.01$, respectively

of the soluble protein concentrations in three types of GS-overexpressed plants compared with wild-type plants (39–54% for *GS1;1*-, 27–51% for *GS1;2*-, and 30–45% for *glnA*-overexpressed plants under normal nutrient solution; 34–46% for *GS1;1*-, 21–27% for *GS1;2*-, and 30–41% for *glnA*-overexpressed plants under low nitrogen nutrient solution; Fig. 2b). There were also 10–20% increases of free NH_4^+ concentrations in three types of GS-overexpressed plants (Fig. 3b) when compared with wild-type plants and negative control plants. In free NO_3^- concentration analysis, 26–33% reduction, 21–29% reduction, and 14–18% increase were observed for *GS1;2*-, *glnA*-, and *GS1;1*-overexpressed plants, respectively (Fig. 3a). The concentrations of total free amino acids were not significantly different in GS-overexpressed plants compared with wild-type plants (data not shown here).

To obtain direct evidence of the enhanced nitrogen assimilation in the GS-overexpressed plants, we also determined the total amino acid concentrations and total nitrogen contents in whole mature plants grown hydroponically in normal or low-nitrogen nutrient solution. The concentrations of all detected amino acids were increased and there was a significant ($P < 0.05$) increase of total amino acid concentrations in the three types of GS-overexpressed plants compared with wild-type plants (13.5% for *GS1;1*-, 6.2% for *GS1;2*-, and 8.6% for *glnA*-overexpressed plants grown in the normal nutrient solution; 37% for *GS1;1*-, 34% for *GS1;2*-, and 22% for *glnA*-overexpressed plants grown in the low nitrogen solution; Fig. 4a). There was also a significant ($P < 0.05$) increase in total nitrogen contents in the three types of GS-overexpressed plants compared with wild-type plants (27–76% for *GS1;1*-, 30–64% for *GS1;2*-, and 50% for *glnA*-overexpressed plants under normal nutrient condition; 25–44% for *GS1;1*-, 13–65% for *GS1;2*-, and 8–18% for *glnA*-overexpressed plants under low nitrogen condition; Fig. 4b).

Yield production and grain amino acid concentration of GS-overexpressed plants

To determine the impact of higher GS mRNA transcriptional levels on yield production, GS-overexpressed and wild-type plants were grown to maturity under normal field conditions.

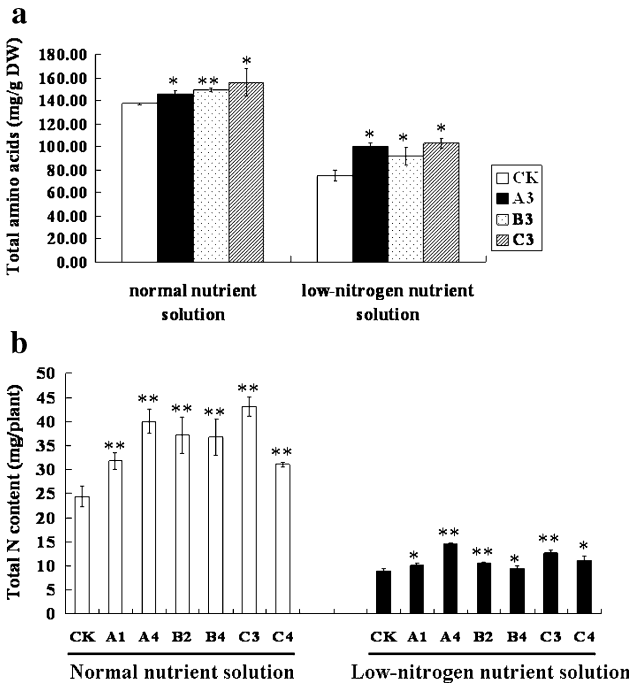

Fig. 4 Higher total amino acids and total nitrogen contents in GS-overexpressed plants. **a** Total amino acids in whole wild-type plants (*CK*) and whole transgenic plants overexpressing *GS1;2* (*A3*), *glnA* (*B3*), and *GS1;1* (*C3*); **b** total nitrogen contents in whole CK and transgenic plants overexpressing *GS1;2* (*A1*, *A4*), *glnA* (*B2*, *B4*), and *GS1;1* (*C3*, *C4*), grown hydroponically in normal nutrient solution (*white bars*) or low-nitrogen nutrient solution (*black bars*) for 4 weeks before harvest. Values are mean ± SD from three independent experiments using six randomly mixed whole plants. *, **Significant differences at the levels of $P = 0.05$ and $P = 0.01$, respectively

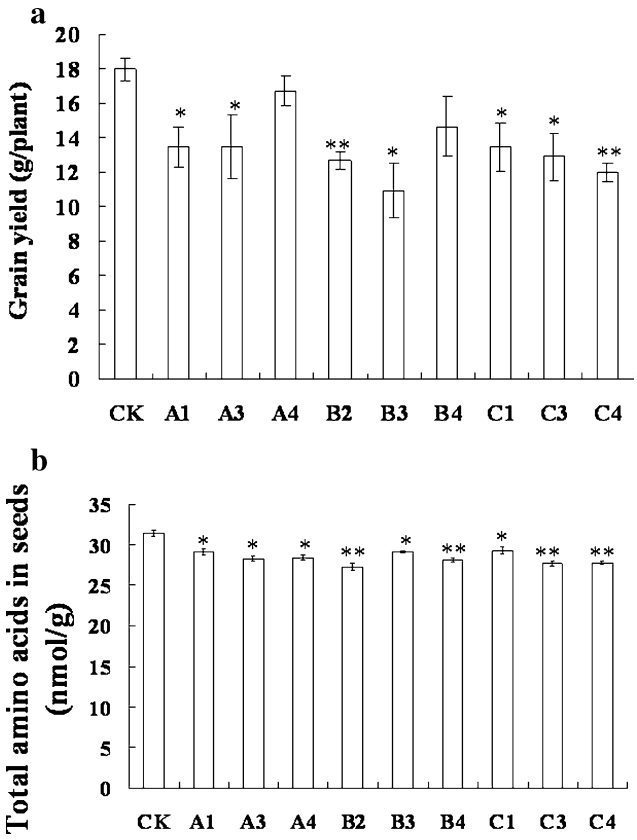

Fig. 5 Lower grain yield production and total amino acids in seed in GS-overexpressed plants. **a** Grain yield production and **b** total amino acids in seed of wild-type plants (*CK*) and transgenic plants overexpressing *GS1;2* (*A1*, *A3*, *A4*), *glnA* (*B2*, *B3*, *B4*), and *GS1;1* (*C1*, *C3*, *C4*), grown under normal field conditions. Values are mean ± SD from more than 24 plants in **a** and two independent experiments using six randomly mixed plant seeds in (**b**). *, **Significant differences at the levels of $P = 0.05$ and $P = 0.01$, respectively

No significant differences were observed in the dry matter production of the vegetative parts of the shoot, which exhibited a normal phenotype in the field. In contrast, significant ($P < 0.05$) decreases in the grain yield were observed when compared with wild-type plants: 25–33% for *GS1;1*-, 7–25% for *GS1;2*-, and 19–39% for *glnA*-overexpressed plants (Fig. 5a). All the detected amino acids in seeds were decreased and there was a significant ($P < 0.05$) reduction of total amino acid concentrations in the three types of GS-overexpressed plants compared with wild-type plants (6.5–11.8% for *GS1;1*-, 7.1–9.9% for *GS1;2*-, and 7.1–13.1% for *glnA*-overexpressed plants; Fig. 5b).

Different responses of the GS-overexpressed plants under abiotic stress conditions

To examine the effect of higher GS mRNA transcriptional levels on resistance to Basta (a phosphinothricin-based herbicide), the seeds of GS-overexpressed, negative control, and wild-type plants in the T_2 generation were germinated on the assay medium [Murashige and Skoog (MS) medium with additional 10 mg/l of Basta). After 7 days, the *GS1;2*-overexpressed seeds showed a significantly high resistance to Basta, and were similar to the wild-type plant seeds that germinated in the MS medium without any Basta and kept growing. The negative control and wild-type plant seeds germinated but failed to show further growth, and some of them died after germination (Fig. 6a). The transgenic plants overexpressing *GS1;1* or *glnA* did not show any resistance to Basta.

We also examined the Basta resistance capacity of the GS-overexpressed seedlings at the two-leaf stage and mature plants at six- to eight-leaf stage by spraying with a 0.5% (v/v) solution of Basta. During subsequent growth and development, the *GS1;2*-overexpressed plants remained healthy with green leaves and stem, whereas the leaves of negative control and wild-type plant turned yellow within 1 week and the plants ultimately died within 2 weeks after spraying (Fig. 6b, c). Thus, both seedlings and mature plants overexpressing *GS1;2* showed high resistance to Basta treatment, whereas the transgenic plants

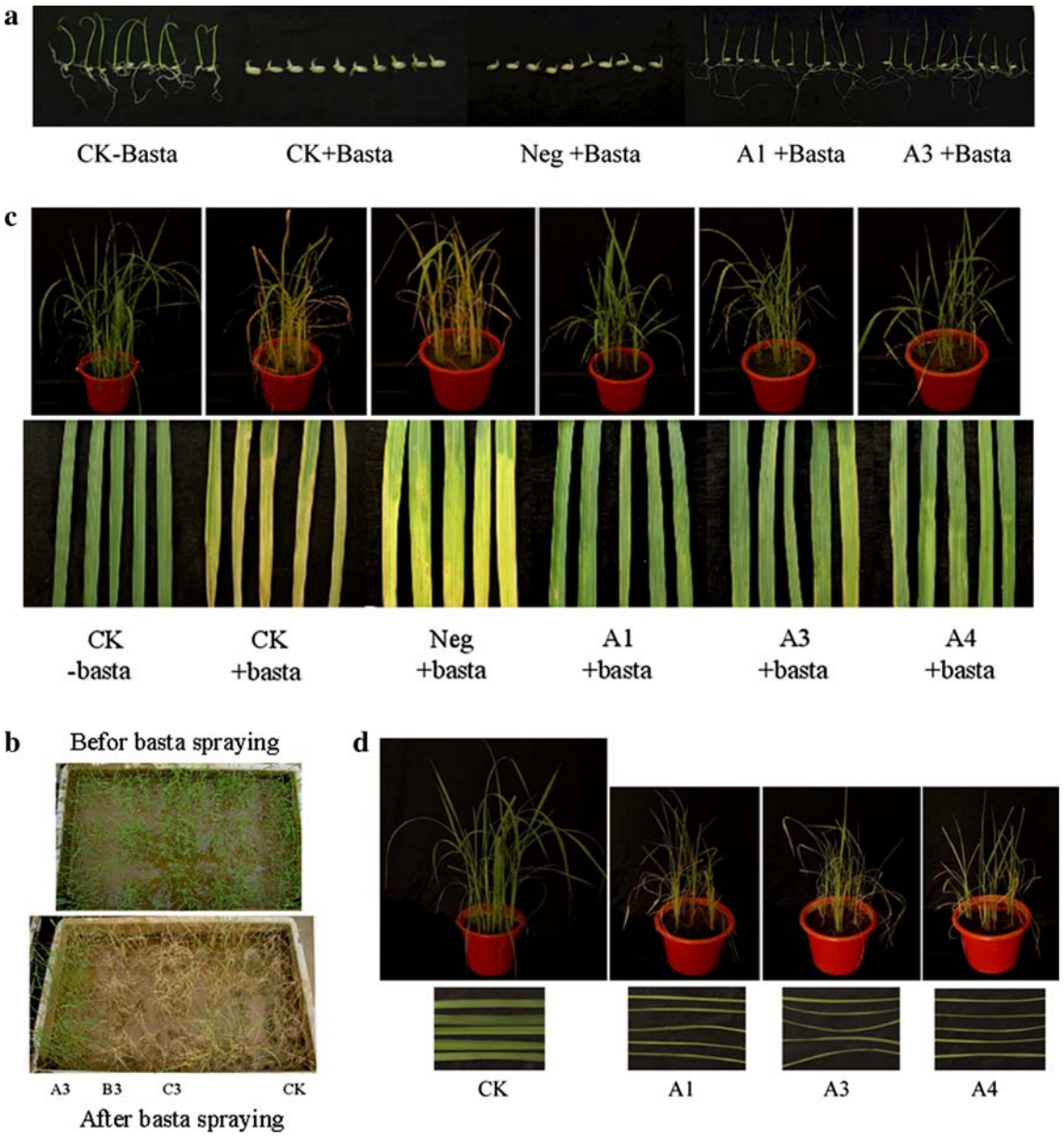

Fig. 6 Phenotypes exhibited under Basta selection and cold stress conditions of *GS1;2*-overexpressed plants. **a** Resistant phenotype exhibited in *GS1;2*-overexpressed plants (*A1*, *A3*) compared with the wild-type plants (*CK*) and negative control plants (*Neg*, transgenic plants with no GS overexpressing) when seeds germinated on the MS medium with 10 mg/l Basta for 2 weeks. **b** Resistant phenotype exhibited in *GS1;2*-overexpressed plants (*A3*) compared with CK at two-leaf stage after Basta spraying for 2 weeks. **c** Resistant phenotype exhibited in mature *GS1;2*-overexpressed plants (*A1*, *A3*, *A4*) compared with mature *CK* and *Neg* after Basta spraying for 2 weeks. **d** Higher sensitivity phenotype exhibited in mature *GS1;2*-overexpressed plants (*A1*, *A3*, *A4*) compared with mature *CK* after exposed at 0–8°C for 2 weeks

overexpressing *GS1;1* and *glnA* did not show any resistance to Basta.

To test the effect of higher GS mRNA transcriptional levels on salt tolerance, the seeds of GS-overexpressed, negative control, and wild-type plants in the T_2 generation were germinated and grown in the assay medium (MS medium with additional 150 mM NaCl) for 3 weeks. Plants at the three-leaf stage were also treated with 200 mM NaCl for 2 days; after 4 weeks of recovery in normal nutrient solution without any NaCl, the root length, plant height, and fresh weight were examined. The root length of transgenic plants did not show any difference compared with the negative control and wild-type plants (data not shown). Both the plant height and fresh weight of *GS1;2*- and *glnA*-overexpressed plants under the NaCl concentration treatments were significantly ($P < 0.05$) lower than the negative control and wild-type plants (20–25% for *GS1;2*-, and 6–27% for *glnA*-overexpressed plants in plant

height; 39–47% for *GS1;2*-, and 20–42% for *glnA*-overexpressed plants in fresh weight), whereas no significant differences were observed in the *GS1;1*-overexpressed plants (Fig. 7a, b). Thus, *GS1;2*- and *glnA*-overexpressed plants but not *GS1;1*-overexpressed plants are sensitive to high salt conditions.

To examine the effect of higher GS mRNA transcriptional levels on drought tolerance, 14-day-old GS-overexpressed, negative control, and wild-type plants in the T_2 generation were grown in a pot with soil. After 12 days of water being withheld, most of the leaves from *GS1;2*-overexpressed plants rolled completely, whereas only a small portion of the leaves of the negative control and wild-type plants rolled slightly. One week after resuming watering, almost 100% of the negative control and wild-type plants recovered, but only 10% of the *GS1;2*-overexpressed plants recovered (data not shown). The transgenic plants overexpressing *GS1;1* and *glnA* did not show any change to the drought condition. Thus, only *GS1;2*-overexpressed plants were highly sensitive to drought conditions compared to the wild-type plants.

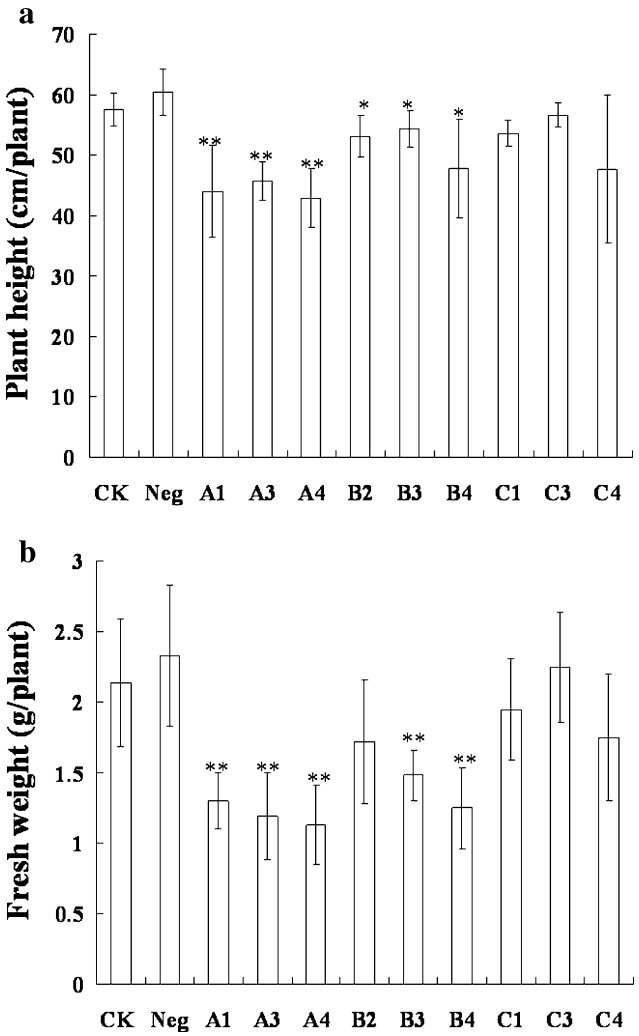

Fig. 7 Plant height and fresh weight of the control plants and GS-overexpressed plants. **a** Plant height and **b** fresh weight of the wild-type plants (*CK*), negative control plants (*Neg*, transgenic plants with no GS overexpressing), and transgenic plants overexpressing *GS1;2* (*A1*, *A3*, *A4*), *glnA* (*B2*, *B3*, *B4*), and *GS1;1* (*C1*, *C3*, *C4*), grown hydroponically in normal nutrient solution with 200 mM NaCl for 2 days and then recovered to a normal nutrient solution without NaCl for 4 weeks before harvest. Values are mean ± SD from 12 plants. *, **Significant differences at the levels of $P = 0.05$ and $P = 0.01$, respectively

To investigate the effect of higher GS mRNA transcriptional levels on cold tolerance, 14-day-old GS-overexpressed, negative control, and wild-type plants in the T_2 generation were treated at 4°C for 3 days. Almost all leaves of the *GS1;2*-overexpressed plants completely rolled, whereas only a small portion of the leaves from the negative control and wild-type plants rolled slightly. In addition, mature GS-overexpressed and wild-type plants in the T_2 generation were also exposed to 0–8°C for 2 weeks. Most *GS1;2*-overexpressed plants died, whereas most wild-type plants were still green and could grow again after shifted to normal conditions (Fig. 6d). The transgenic plants overexpressing *GS1;1* and *glnA* did not show any change to the cold stress condition. Again, only *GS1;2*-overexpressed plants had a higher sensitivity to stress conditions.

Discussion

Phenotypes exhibited in GS-overexpressed rice plants

Nitrogen assimilation plays an important role in the growth and development of plants. The first step of incorporation of inorganic nitrogen into organic nitrogenous compounds by the GS/GOGAT cycle is likely to be a major checkpoint for controlling assimilation of plant nitrogen. Because of its importance in nitrogen assimilation for crop plants, numerous groups have attempted to improve nitrogen assimilation by biochemical and molecular biological methods, especially the genetic transformation of *GS* genes. Such as transgenic lotus plants (Vincent et al. 1997), poplar trees (Gallardo et al. 1999; Pascual et al. 2008), tobacco (Fuentes et al. 2001; Oliveira et al. 2002) and wheat (Habash et al. 2001). Here, we report studies in which *GS1;1*, *GS1;2*, and *glnA* were overexpressed in rice transgenic plants to test the effects of this manipulation on primary nitrogen assimilation and plant growth. The fresh weight and dry weight of three GS-overexpressed plants were slightly higher but not significantly different than those of wild-type plants. Similar results were also observed in transgenic alfalfa (Ortega et al. 2001) and transgenic maize (Martin et al. 2006) overexpressing GS1 under a

CaMV 35S promoter, and transgenic rice expressing *GS1;1* under the control of its own promoter (Tabuchi et al. 2007), which exhibited no difference in growth phenotype.

Metabolic impacts of increased GS mRNA transcripts

The accumulation of GS mRNA transcripts in three types of GS-overexpressed plant leaves showed 18–25% higher total GS activities and 27–54% higher soluble protein concentrations under normal nutrient condition, and 36–46% higher total GS activities and 21–46% higher soluble protein concentrations under low-nitrogen condition. Similar results were also found in transgenic tobacco (Oliveira et al. 2002), peas (Fei et al. 2003), and maize (Martin et al. 2006) overexpressing GS1.

The free NO_3^- level in leaves showed a 21–33% decrease in *GS1;2*- and *glnA*-overexpressed plants and a 14–18% increase in *GS1;1*-overexpressed plants. A 10–20% increase in free NH_4^+ level in the three types of GS-overexpressed plants was observed, which was opposite to results in transgenic tobacco, which had 6.3- to 7-fold reduction in the total levels of free NH_4^+ (Oliveira et al. 2002). The different results for free NO_3^- in the GS-overexpressed plants may indicate distinct roles of *GS1;1* and *GS1;2* in nitrogen assimilation. GS1;2 is highly expressed in roots and mainly functions in primary NH_4^+ assimilation, while GS1;1 is highly expressed in shoot phloem tissues and therefore functions in translocating nitrogenous compounds to the developing sink tissues. Higher GS1;2 mRNA transcripts in *GS1;2*-overexpressed plants can accelerate the reduction from NO_3^- to NH_4^+ to decrease the nitrate level in cells, and higher GS1;1 mRNA transcripts in *GS1;1*-overexpressed plants can accelerate the translocation of NO_3^- and NH_4^+ from root to leaf to increase both the NO_3^- and NH_4^+ levels. The increased total levels of leaf free NO_3^- and NH_4^+ in *GS1;2*- and *glnA*-overexpressed plants could be due to the higher GS mRNA transcripts and total GS activities, which can accelerate the nitrogen absorption and reduction ability in plants.

Analysis of leaf free amino acids showed no obvious changes in total amino acid contents in the three types of GS-overexpressed plants. In transgenic lotus, however, a large increase in the amino acid contents of roots and shoots was observed (Vincent et al. 1997). In the whole plant, higher amino acid contents and total nitrogen contents were observed in the GS-overexpressed plants. Similar results were also found in GS-overexpressed transgenic poplar plants characterized by enhanced nitrogen uptake (Pascual et al. 2008); transgenic peas showed a 30% increase in nitrogen accumulation under 0.1 mM NO_3^- conditions (Fei et al. 2003); and transgenic wheat demonstrated an enhanced capacity to accumulate nitrogen (Habash et al. 2001).

Several studies have demonstrated a direct correlation between enhanced GS activities and yield production in transgenic plants. Transgenic wheat lines expressing *P. vulgaris GS1* under the control of rice *rbc*S promoter were grown in pots to maturity; the plants showed significantly higher grain yield and grain nitrogen content (Habash et al. 2001). Transgenic maize overexpressing GS1 showed a ~30% increase in grain yield when compared with two corresponding wild-type control plants (Martin et al. 2006). However, we observed a 7–39% decrease in yield production and ~10% decrease in seed amino acid content in GS-overexpressed plants. The *GS* genes studied here may be functioning only in rice vegetative growth in the GS-overexpressed plants, higher GS activities increased the plant metabolic level and may accelerate plant growth, which could accelerate the leaf senescence, break the nitrogen compounds translocation and reassimilation in the plant developmental stages. As less energy and nutrient compounds can be used by the GS-overexpressed plants during reproductive stage, this would be detrimental to grain yield and lead to a significant decrease in yield production and seed amino acid contents. Also, the constitutive highly expressed 35S promoter used in this study could be another reason for the decreased yield production and seed amino acid contents. Higher expression of GS mRNA in all tissues and organs in the GS-overexpressed plants would disturb the normal nutrient metabolic processes and signaling pathways arising some problems in remobilization of nitrogen, carbon, and minerals from source and sink during seed development. Similarly, transgenic lotus plants overexpressing soybean GS1 driven by a *CaMV* 35S promoter exhibited accelerated growth rate and leaf senescence (Vincent et al. 1997), and an increase in the activity of GS was observed during leaf senescence (Feller and Fischer 1994). The physiological parameters relevant to seed yields and seed nitrogen contents include the efficiency of nitrogen assimilation or re-assimilation in vegetative tissues as well as the remobilization of nitrogen during flowering and seed maturation. Several researchers have identified genes encoding proteins that are specifically activated during the remobilization of nitrogen, carbon, and minerals during leaf senescence (e.g., Gallais and Hirel 2004). In addition, efforts are being made to study the biochemical mechanisms involved in nitrogen export and import from source and sink during senescence (Hayakawa et al. 1994; Masclaux et al. 2000). In spite of the studies conducted at the whole plant level and using transgenic plants, our understanding of the mechanisms involved in nitrogen remobilization during leaf senescence is still at a preliminary stage.

Different behaviors of GS-overexpressed plants under abiotic stress conditions

Although numerous biochemical and physiological studies have been concentrated on GS-overexpressed plants, fewer studies have investigated the performance of GS-overexpressed plants under abiotic stress conditions. In the present study, three types of GS-overexpressed plants were exposed to Basta, salt, drought, and cold stresses.

Glutamine synthetase is the key enzyme involved in ammonia assimilation in plants and is the target of phosphinothricin [L-2-amino-4-(hydroxymethylphosphinyl) butanoic acid (PPT)], a substrate analog of glutamate. PPT is used as the main active component of Basta, an herbicide commonly used for weed control in agriculture. As a result of the inhibition of GS, PPT blocks photorespiration, resulting in the depletion of leaf amino acid pools and leading to the plant death. Therefore, overexpressing GS in plants would be a useful method to improve Basta resistance. Transgenic wheat (Huang et al. 2005) and rice plants (Sun et al. 2005) overexpressing both GS1 and GS2 showed Basta-tolerant properties. Transgenic poplar overexpressing cytosolic GS1 exhibited enhanced resistance to PPT when compared with nontransgenic controls (Pascual et al. 2008). Similarly, in this study Basta resistance was observed in *GS1;2*-overexpressed rice plants at three developmental stages (seed germination, two-leaf seedling, and mature plant stages). However, Basta resistance was not observed in *GS1;1*- or *glnA*-overexpressed rice plants at any developmental stage. This appears to indicate the differential physiological roles of GS1;1 and GS1;2 in ammonium assimilation in plants. The higher GS1;2 mRNA transcriptional level in *GS1;2*-overexpressed plants can decrease the toxicity of PPT, which may be sufficient to assimilate ammonium to produce glutamine to maintain plant growth. Our study suggest that PPT resistance is conferred by effective overexpression of GS1;2 in transformed rice plants, and *GS1;2* can serve as a selective marker gene instead of *bar* gene for the selection of PPT in transformation systems.

Higher sensitivity to salt, drought, and cold stresses were observed in the *GS1;2*-overexpressed rice plants, whereas *GS1;1*- and *glnA*-overexpressed plants displayed no significant differences phenotype compared with wild-type plants. These results contrast to those of previous studies: overexpressing GS2 in rice resulted in increased salt and cold tolerance (Hoshida et al. 2000) and transgenic poplar plants overexpressing cytosolic GS1 were characterized by enhanced tolerance to water stress (Pascual et al. 2008). Salt and water stresses can increase the proteolytic activity and decrease the protein synthesis in cells, which causes an accumulation of free amino acids. This, in turn, might reduce the need for ammonium incorporation to form amino acids and indirectly cause the excessive accumulation of ammonium. Thus, under stress conditions the higher GS1;2 mRNA transcriptional level in *GS1;2*-overexpressed plants may induce more ammonium absorption in roots, leading to a greater accumulation of ammonium, which may be toxic and induce cell senescence. The different tolerances of the three types of GS-overexpressed plants suggest the distinct roles of GS1;1 and GS1; 2 in nitrogen assimilation and abiotic stress mechanism in plants, although more detailed analyses are needed.

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